OECD 429
IUCLID OK

[redacted]

[redacted]

LOCAL LYMPH NODE ASSAY (LLNA)

IN MICE

WITH

[redacted]

REPORT

Study Completion Date:

[redacted]

1 COPY OF GLP-CERTIFICATE

[redacted]

HESSEN

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

[redacted]

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according chemVwV-GLP Nr. 5.3/OECD guidance)

2 Prüfungen zur Bestimmung der toxikologischen Eigenschaften	**2** Toxicity studies
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)	**3** Mutagenicity studies
6 Prüfungen zur Bestimmung von Rückständen	**6** Residues
8 Analytische Prüfungen an biologischen Materialien	**8** Analytical studies on biological materials

15.08. und 27. – 29.10.2008
Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP- Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Im Auftrag

Th. Zimmermann, Referent, Wiesbaden, den **30. März 2009**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

HESSISCHES MINISTERIUM FÜR UMWELT, LÄNDLICHEN RAUM UND VERBRAUCHERSCHUTZ 2

Hess. Ministerium für Umwelt, Energie, Landwirtschaft und Verbraucherschutz,
Mainzer Straße 80 D65189 Wiesbaden
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority

2 CONTENTS

3 PREFACE

3.1 General

Title: Local Lymph Node Assay (LLNA) in Mice with [redacted]

Sponsor: [redacted]

Study Monitor:

Test Facility:

3.2 Responsibilities

Study Director: [redacted]

Deputy Study Director:

Management:

Head of Quality Assurance Unit:

3.3 Schedule

Experimental Starting Date: [redacted]

Experimental Completion Date:

3.4 Project Staff Signature

Study Director [redacted]

3.5 Good Laboratory Practice

The study was performed in compliance with:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), in its currently valid version.

"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final]

3.6 Guidelines

This study followed the procedures indicated by the following internationally accepted guidelines and recommendations:

OECD Guidelines for Testing of Chemicals, Updated Guideline 429: Skin Sensitisation: Local Lymph Node Assay (adopted 22 July 2010).

"European Union Method B.42, Skin Sensitisation: Local Lymph Node Assay - Commission regulation (EC) No 440/2008, Official Journal of the European Union (EN), dated May 30, 2008".

3.7 Archiving

[redacted] will archive:

Raw data, study plan, report, and specimens (if any) for at least 3 years at the test facility's archive. Thereafter, the material will be transferred to the GLP archive of [redacted] for archiving the remaining time up to a total archiving period of 15 years. No data will be discarded without the sponsor's written consent. A sample of the test item will be archived two years after the expiration date provided by the sponsor. Thereafter the samples will be discarded without further notice.

3.8 Deviations from the Study Plan

The Internal Test Item Number used for the pre-experiment was [redacted] and for the main experiment [redacted]

During the acclimation phase, the relative humidity in the animal room was between approximately 31 – 65% for a maximum of 12 hours.

The catalogue number of trichloroacetic acid changed from [redacted] to [redacted]

These deviations to the study plan, however, did not affect the validity of the study.

4 STATEMENT OF COMPLIANCE

[redacted] [redacted]

Test Item:

Study Director: [redacted]

Title: Local Lymph Node Assay (LLNA) in Mice with [redacted]

This study performed in the test facility of [redacted] was conducted in compliance with Good Laboratory Practice Regulations:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), in its currently valid version.

"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final]

There were no circumstances that may have affected the quality or integrity of the study.

Study Director [redacted]

5 STATEMENT OF QUALITY ASSURANCE UNIT

[redacted] [redacted]

Test Item:

Study Director: [redacted]

Title: Local Lymph Node Assay (LLNA) in Mice with [redacted]

The general facilities and activities of [redacted] are inspected periodically and the results are reported to the responsible person and the Management.

Study procedures were inspected periodically. The study plan and this report were audited by the Quality Assurance Unit. The dates are given below.

Phases and Dates of QAU Inspections/ Audits		Dates of Reports to the Study Director and to Management
Study Plan:		[redacted]
1st Amendment to Study Plan:		
Process Inspection preparation for application:		
Report:		

This statement is to confirm that the present report reflects the raw data.

Head of Quality Assurance Unit [redacted]

6 SUMMARY

In this study the test item [redacted] suspended in dimethylformamide (DMF) was assessed for its possible contact allergenic potential.

For this purpose a local lymph node assay was performed using test item concentrations of 10, 25, and 50% (w/v). The highest concentration tested was the highest concentration that could be achieved whilst avoiding systemic toxicity and excessive local skin irritation as confirmed by a pre-experiment.

The animals did not show any signs of systemic toxicity or local skin irritation during the course of the study and no cases of mortality were observed.

In this study Stimulation Indices (S.I.) of 1.21, 1.87, and 1.81 were determined with the test item at concentrations of 10, 25, and 50% (w/v) in DMF, respectively.

The test item [redacted] was **not a skin sensitiser** under the test conditions of this study.

7 OBJECTIVE

7.1 Aims of the Study

The purpose of this Local Lymph Node assay was to identify the contact allergenic potential of [redacted] when administered to the dorsum of both ears of mice.

This study should provide a rational basis for risk assessment to the sensitising potential of the test item in man.

7.2 Outline of the Performed Study

In order to study a possible allergenic potential of [redacted] three groups each of four female mice were treated with different concentrations of the test item by topical application at the dorsum of each ear once daily each on three consecutive days. A control group of four mice was treated with the vehicle only. Five days after the first topical application, the mice were intravenously injected into a tail vein with radio-labelled thymidine (^{3}H-methyl thymidine). Approximately five hours after intravenous injection, the mice were sacrificed and the draining auricular lymph nodes excised and pooled per group. Single cell suspensions of lymph node cells were prepared from pooled lymph nodes, which were subsequently washed and incubated with trichloroacetic acid overnight. The proliferative capacity of pooled lymph node cells was determined by the incorporation of ^{3}H-methyl thymidine measured in a β-scintillation counter.

8 MATERIALS AND METHODS

8.1 Test Item

Internal Test Item Number: [redacted]

The test item and the information concerning the test item were provided by the sponsor.

Identity: [redacted]
Batch No.:
Purity:
Stability in solvent:
Storage:
Expiration Date:

8.2 Vehicle and Dose Selection

A solubility experiment was performed according to the recommendations given by OECD 429. The highest test item concentration, which could be technically used was a [redacted]n dimethylformamide after vortexing.

To determine the highest non-irritant test concentration that did at the same time not induce signs of systemic toxicity, a pre-test was performed in two animals and stated in raw data and report. Two mice were treated by (epidermal) topical application to the dorsal surface of each ear with test item concentrations of 25 and 50% once daily each on three consecutive days. Prior to the first application of the test item and before sacrifice the body weight was determined. Clinical signs were recorded at least once daily. Eventual signs of local irritation were documented and a score was used to grade a possible reddening of the ear skin. Furthermore, prior to the first application of the test item (day 1), on day 3 and before sacrifice (day 6) the ear thickness was determined using a micrometer (S0247 Kroeplin, 36381 Schlüchtern, Germany). Additionally, for both animals, the ears were punched after sacrifice (day 6) at the apical area using a biopsy punch (Stiefel, Ø 8 mm corresponding to 0.5 cm^2) and were immediately pooled per animal and weighed using an analytical balance (for individual results see Annex 1). Eventual ear irritation was considered to be excessive if reddening of the ear skin of a score value ≥3 was observed at any observation time and/or if an increase in ear thickness of ≥25% was recorded on day 3 or day 6. At the tested concentrations, the animals did not show any signs of local skin irritation or systemic toxicity.

Therefore, the test item in the main study was assayed at 10, 25, and 50 % (w/v). The highest concentration tested was thus the highest level that could be achieved whilst avoiding systemic toxicity and excessive local irritation in the pre-experiment.

8.3 Test Item Preparation

The test item was placed into an appropriate container on a tared balance and DMF was added. The different test item concentrations were prepared individually. Homogeneity of the test item in vehicle was maintained during treatment using a magnetic stirrer.

The preparations were made freshly before each dosing occasion.

Concentrations were in terms of material as supplied.

8.4 Chemicals

^{3}H-Methyl thymidine	Hartmann Analytic (MT6032, aqueous solution) 74 GBq/mmol (2 Ci/mmol), 37 MBq/mL (1 mCi/mL)
Trichloroacetic acid	Merck 1.00807.1000 (min. 99.5 %)
Phosphate buffered saline	Fluka no. 79382 (1 tablet solved in 200 mL deionised water)

8.5 Vehicle

Dimethylformamide (DMF)

Manufacturer	Merck KGaA (64293 Darmstadt, Germany)
Supplier	VWR International GmbH (64295 Darmstadt, Germany)
Purity	99%
Catalogue number	8.22275.1000
Batch number	K38049575-749
Storage conditions	In the original container at room temperature (20°C ± 5°C), away from direct sunlight.

9 TEST SYSTEM

9.1 Animal Species

Test system	Mice, CBA/CaOlaHsd
Rationale	Recognised as the recommended test system
Source	[redacted]
Number of animals for the pre-test	2 females
Number of animals for the main study	16 females
Number of animals per group	4 females (nulliparous and non-pregnant)
Number of test groups	3
Number of control (vehicle) groups	1
Age	8 - 12 weeks (beginning of treatment)
Body weight	See Annex 1
Identification	The animals were distributed into the test groups at random. All animals belonging to the same experimental group were kept in one cage. The animals were identified by tail tags. In the pre-experiment, animals were identified by cage number.
Acclimatisation	At least 5 days prior to the start of dosing under test conditions after health examination. Only animals without any visible signs of illness were used for the study.

9.1.1 Husbandry

The animals were kept conventionally. The experiment was conducted under standard laboratory conditions.

Housing:	Group
Cage Type:	Makrolon Type II / III, with wire mesh top (EHRET GmbH, 79302 Emmendingen, Germany)
Bedding:	granulated soft wood bedding (Rettenmaier & Söhne GmbH + Co. KG, 73494 Rosenberg, Germany)
Feed:	pelleted standard diet, ad libitum [redacted]
Water:	tap water, ad libitum (Gemeindewerke, 64380 Rossdorf, Germany)
Environment:	temperature 22 ± 2°C relative humidity 31-65% artificial light 6.00 a.m. - 6.00 p.m.

9.2 Allocation

The animals were distributed as follows:

Group	Concentration[b] (%)	Number of Animals per Group	Animal Numbers (Group Housing)
1 (Control Group[a])	—	4	1 - 4
2 (Low Dose)	10	4	5 - 8
3 (Mid Dose)	25	4	9 - 12
4 (High Dose)	50	4	13 - 16

a) vehicle group = dimethylformamide (DMF)

b) concentrations as determined in a pre-experiment.

9.3 Experimental Design and Procedures

9.3.1 Topical Application

Each test group of mice was treated by topical (epidermal) application to the dorsal surface of each ear with test item concentrations of 10, 25, and 50% (w/v) in DMF. The application volume, 25 µL/ear/day, was spread over the entire dorsal surface (∅ ~ 8 mm) of each ear once daily for three consecutive days. A further group of mice was treated with an equivalent volume of the relevant vehicle alone (control animals).

9.3.2 Administration of ^{3}H-Methyl Thymidine

^{3}H-methyl thymidine (^{3}HTdR) was purchased from Hartmann Analytic, 38124 Braunschweig, Germany (specific activity, 2 Ci/mmol; concentration, 1 mCi/mL).

Five days after the first topical application (day 6) 250 µL of phosphate-buffered saline (PBS) containing 19.7 µCi of ^{3}HTdR (equivalent to ^{3}HTdR 78.6 µCi/mL) were injected into each test and control mouse via the tail vein.

9.3.3 Determination of Incorporated ^{3}HTdR

Approximately five hours after treatment with ^{3}HTdR all mice were euthanised by intraperitoneal injection of Pentobarbital-Natrium (Release®, WDT, 30827 Garbsen, Germany).

The draining lymph nodes were rapidly excised and pooled per group (8 nodes per group). Single cell suspensions (in phosphate buffered saline) of pooled lymph node cells were prepared by gentle mechanical disaggregation through stainless steel gauze (200 µm mesh size). After washing two times with phosphate buffered saline (approx. 10 mL) the lymph node cells were resuspended in 5 % trichloroacetic acid (approx. 3 mL) and incubated at approximately +4 °C for at least 18 hours for precipitation of macromolecules. The precipitates were then resuspended in 5 % trichloroacetic acid (1 mL) and transferred to plastic scintillation vials with 10 mL of 'Ultima Gold' scintillation liquid (Perkin Elmer (LAS) GmbH, 63110 Rodgau, Germany) and thoroughly mixed.

The level of ^{3}HTdR incorporation was then measured on a β-scintillation counter (Tricarb 2900 TR, Perkin Elmer (LAS) GmbH, 63110 Rodgau, Germany). Similarly, background ^{3}HTdR levels were also measured in two 1ml-aliquots of 5 % trichloroacetic acid. The β-scintillation counter expresses ^{3}HTdR incorporation as the number of radioactive disintegrations per minute (DPM).

9.3.4 Interpretation of Raw Data

The proliferative response of lymph node cells is expressed as the number of radioactive disintegrations per minute per lymph node (DPM/node) and as the ratio of ^{3}HTdR incorporated into lymph node cells of test lymph nodes relative to that recorded for control lymph nodes (Stimulation Index; S.I.). Before DPM/node values were determined, mean scintillation-background DPM was subtracted from test and control raw data.

A test item is regarded as a sensitiser in the LLNA if the following criteria are fulfilled:

- First, that exposure to at least one concentration of the test item resulted in an incorporation of ^{3}HTdR at least 3-fold or greater than that recorded in control mice, as indicated by the stimulation index.
- Second, that the data are compatible with a conventional dose response, although allowance must be made (especially at high topical concentrations) for either local toxicity or immunological suppression.

9.4 Observations

In addition to the sensitising reactions the following observations and data were recorded during the test and observation period:

Mortality / Viability	Once daily from experimental start to necropsy.
Body weights	In the pre-test prior to the first application and prior to sacrifice. In the main experiment: prior to the first application and prior to treatment with ^{3}HTdR.
Ear thickness	In the pre-test prior to the first application of the test item (day 1), on day 3 and before sacrifice (day 6).
Ear weights	In the pre-test after sacrifice; biopsy punches were taken from each ear.
Clinical signs (local / systemic)	Clinical signs were recorded at least once daily. Especially the treatment sites were observed carefully.

9.5 Statistical Analysis

The mean values and standard deviations were calculated in the body weight tables.

9.6 Positive Control Data

The sensitivity and reliability of the experimental technique employed was assessed by use of a substance which is known to have skin sensitisation properties in CBA/CaOlaHsd mice. The periodic positive control experiment was performed with α-hexyl cinnamaldehyde in acetone:olive oil (4:1 v/v) using CBA/CaOlaHsd mice in [redacted] see Annex 2.

10 RESULTS

10.1 Calculation and Results of Individual Data

Vehicle: dimethylformamide

Test item concentration % (w/v)	Group	Measurement DPM	Calculation			Result
			DPM-BG[a)]	number of lymph nodes	DPM per lymph node[b)]	S.I.
---	BG I	18	---	---	---	---
---	BG II	41	---	---	---	---
0	1	2597	2568	8	320.9	**1.00**
10	2	3139	3110	8	388.7	**1.21**
25	3	4842	4813	8	601.6	**1.87**
50	4	4676	4647	8	580.8	**1.81**

BG = Background (1 ml 5% trichloroacetic acid) in duplicate

1 = Control Group

2-4 = Test Groups

S.I. = Stimulation Index

a) = The mean value was taken from the figures BG I and BG II

b) = Since the lymph nodes of the animals of a dose group were pooled, DPM/node was determined by dividing the measured value by the number of lymph nodes pooled

The EC3 value could not be calculated, since all S.I.´s are below the threshold value of 3.

10.2 Viability / Mortality

No deaths occurred during the study period.

10.3 Clinical Signs

The animals did not show any signs of systemic toxicity or local skin irritation during the course of the study.

10.4 Body Weights

The body weight of the animals, recorded prior to the first application and prior to treatment with ^{3}HTdR, was within the range commonly recorded for animals of this strain and age.

The individual body weight values are included in Annex 1.

11 DISCUSSION

In order to study a possible contact allergenic potential of [redacted] three groups each of four female mice were treated once daily with the test item at concentrations of 10, 25, and 50% (w/v) in DMF by topical application to the dorsum of each ear for three consecutive days. The highest concentration tested was the highest concentration that could be achieved whilst avoiding systemic toxicity and excessive local skin irritation as confirmed by a pre-experiment. A control group of four mice was treated with the vehicle (DMF) only. Five days after the first topical application the mice were injected intravenously into a tail vein with radio-labelled thymidine (^{3}H-methyl thymidine). Approximately five hours after intravenous injection, the mice were sacrificed, the draining auricular lymph nodes excised and pooled per group. Single cell suspensions of lymph node cells were prepared from pooled lymph nodes, which were subsequently washed and incubated with trichloroacetic acid overnight. The proliferative capacity of pooled lymph node cells was determined by the incorporation of ^{3}H-methyl thymidine measured in a β-scintillation counter.

All treated animals survived the scheduled study period and no signs of systemic toxicity or local skin irritation were observed.

A test item is regarded as a sensitiser in the LLNA if the exposure to one or more test concentration resulted in a 3-fold or greater increase in incorporation of ^{3}HTdR compared with concurrent controls, as indicated by the Stimulation Index (S.I.). The estimated concentration of test item required to produce a S.I. of 3 is referred to as the EC3 value.

In this study Stimulation Indices of 1.21, 1.87, and 1.81 were determined with the test item at concentrations of 10, 25, and 50% in DMF. A clear dose response was not observed. An EC3 value could not be calculated, since none of the tested concentrations induced a S.I. greater than the threshold value of 3.

11.1 CONCLUSION

The test item [redacted] was **not a skin sensitiser** under the test conditions of this study.

12 REFERENCES

1) OECD Guidelines for Testing of Chemicals, Updated Guideline 429: Skin Sensitisation: Local Lymph Node Assay (adopted 22 July 2010).

2) Kimber I., Hilton J. and Weisenberger C. (1989). The murine local lymph node assay for identification of contact allergens: a preliminary evaluation of in situ measurement of lymphocyte proliferation. Contact Dermatitis, 21, 215-220.

3) Kimber I. and Basketter D.A. (1992). The murine local lymph node assay. A commentary on collaborative studies and new directions. Food and Chemical Toxicology, 30, 165-169.

4) Basketter D.A., Gerberick G.F., Kimber I. and Loveless S.E. (1996). The local lymph node assay: a viable alternative to currently accepted skin sensitization tests. Food and Chemical Toxicology, 34, 985-997.

5) Chamberlain M. and Basketter D.A. (1996). The local lymph node assay: status of validation. Food and Chemical Toxicology, 34, 999-1002.

6) Basketter D.A., Lea L.J., Cooper K., Stocks J., Dickens A., Pate I., Dearman R.J. and Kimber I. (1999). Threshold for Classification as a Skin Sensitizer in the Local Lymph Node Assay: a Statistical Evaluation. Food and Chemical Toxicology, 37, 1-8.

7) Steiling W., Basketter D.A., Berthold K., Butler M., Garrigue J-L., Kimber I., Lea L.J., Newsome C., Roggeband R., Stropp G., Waterman S. and Wiemann C. (2001): Skin Sensitisation Testing - New Perspectives and Recommendations. Food and Chemical Toxicology, 39, 293-301.

13 DISTRIBUTION OF THE REPORT

Sponsor	1x copy, 1x PDF
Study Director	1x original

14 ANNEX 1

14.1 Results of the Pre-Test

Body Weights

Animal No.	Concentration % (w/v)	Body Weight			
		prior 1st Application (g)	prior to sacrifice (Day 6) (g)	Difference Day 1 to Day 6 (g)	Difference %
1	25	18.0	20.4	2.4	13.3
2	50	19.6	21.1	1.5	7.7

Ear Thickness

Animal No:	Conc. % (w/v)	Ear Thickness								
		prior to 1st Application (µm)			prior to 3rd Application (µm)			prior to Necropsy (µm)		
		Right Ear	Left Ear	Mean	Right Ear	Left Ear	Mean	Right Ear	Left Ear	Mean
1	25	250	250	250.0	255	250	252.5	265	255	260.0
2	50	255	250	252.5	255	255	255.0	275	270	272.5

Difference Day 1 to Day 3 (µm)	Ear Swelling Day 3 (%)	Difference Day 1 to Day 6 (µm)	Ear Swelling Day 6 (%)
2.5	1.0	10.0	4.0
2.5	1.0	20.0	7.9

Ear Weights

Animal No.	Concentration % (w/v)	**Ear Weights after Necropsy** (mg per animal)
1	25	34.47
2	50	29.98

14.2 Tables of Body Weights

Individual animal weights at the start of the experiment

Dose Group	Animal No.	Initial Weight (g)	Mean SD	Range
Negativ Control	1	20.0		
DMF	2	21.7		
	3	19.7		
	4	23.1	21.1 ± 1.6	23.1 - 19.7
Test Item	5	22.4		
Dose: 10.000 [%]	6	22.8		
PS 1 / 10%	7	22.3		
	8	22.0	22.4 ± 0.3	22.8 - 22.0
Test Item	9	21.0		
Dose: 25.000 [%]	10	20.7		
PS 2 / 25%	11	20.0		
	12	21.5	20.8 ± 0.6	21.5 - 20.0
Test Item	13	21.9		
Dose: 50.000 [%]	14	21.3		
PS 3 / 50%	15	22.2		
	16	20.4	21.5 ± 0.8	22.2 - 20.4
Summary			21.4 ± 1.0	19.7 - 23.1

Individual animal weights prior administration of ^{3}H-methyl thymidine

Dose Group	Animal No.	Initial Weight (g)	Mean SD	Range
Negativ Control	1	20.7		
DMF	2	22.0		
	3	23.3		
	4	24.3	22.6 ± 1.6	24.3 - 20.7
Test Item	5	23.4		
Dose: 10.000 [%]	6	23.6		
PS 1 / 10%	7	21.9		
	8	23.9	23.2 ± 0.9	23.9 - 21.9
Test Item	9	22.7		
Dose: 25.000 [%]	10	21.3		
PS 2 / 25%	11	21.1		
	12	21.7	21.7 ± 0.7	22.7 - 21.1
Test Item	13	23.4		
Dose: 50.000 [%]	14	22.0		
PS 3 / 50%	15	23.1		
	16	21.3	22.5 ± 1.0	23.4 - 21.3
Summary			22.5 ± 1.1	20.7 - 24.3

15 ANNEX 2

15.1 Results of the GLP Positive Control

Experiment performed in [redacted]

Positive control substance: α-hexyl cinnamaldehyde

Vehicle: acetone:olive oil (4:1 v/v)

Test item concentration % (w/v)	Group	Measurement DPM	Calculation			Result
			DPM-BG [a)]	number of lymph nodes	DPM per lymph node [b)]	**S.I.**
---	BG I	15	---	---	---	---
---	BG II	11	---	---	---	---
0	1	2291	2278	8	284.8	**1.00**
5	2	2421	2408	8	301.0	**1.06**
10	3	6596	6583	8	822.9	**2.89**
25	4	14711	14698	8	1837.3	**6.45**

BG = Background (1 ml 5% trichloroacetic acid) in duplicate

1 = Control Group

2-4 = Test Group

S.I. = Stimulation Index

a) = The mean value was taken from the figures BG I and BG II

b) = Since the lymph nodes of the animals of a dose group were pooled, DPM/node was determined by dividing the measured value by the number of lymph nodes pooled

	Test item concentration %	S.I.
Test Group 3	10 (a)	2.89 (b)
Test Group 4	25 (c)	6.45 (d)
EC3 = (a-c) [(3-d)/(b-d)] + c = **10.5 %** (w/v)		

EC3 = Estimated concentration for a S.I. of 3.

a,b,c,d = Co-ordinates of the two pairs of data lying immediately above and below the S.I. value of 3 on the LLNA dose response plot.

15.2 Historical Positive Control Data

These values represent the data of 10 consecutive positive controls ([redacted]

	Stimulation Index at 25% (w/v) (α-HCA in acetone:olive oil (4:1 v/v))
Mean ± SD	6.31 ± 1.34
No. of experiments	10